



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 15/00, C12P 21/02 C07K 7/10, C07H 21/00	A1	(11) International Publication Number: WO 89/ 05857 (43) International Publication Date: 29 June 1989 (29.06.89)
(21) International Application Number: PCT/SE88/00696 (22) International Filing Date: 21 December 1988 (21.12.88) (31) Priority Application Number: 8705139-7 (32) Priority Date: 23 December 1987 (23.12.87) (33) Priority Country: SE (71) Applicant (for all designated States except US): TRION FORSKNING- OCH UTVECKLINGSS AKTIEBO- LAG [SE/SE]; Aspvägen 1A, S-191 41 Sollentuna (SE). (72) Inventors; and (75) Inventors/Applicants (for US only) : BARTFAI, Tamas [SE/SE]; Brinken 3, S-182 74 Stocksund (SE). TJÖRNHAMMAR, Marie-Louise [SE/SE]; SI- MONCSITS, András [HU/SE]; Tavastgatan 11, S-117 24 Stockholm (SE). KÁLMÁN, Miklós [HU/HU]; Jakab, L. u. 12, H-6726 Szeged (HU). CSERPÁN, Im- re [HU/HU]; Szamosu. 1/a, H-6723 Szeged (HU).	(74) Agent: AWAPATENT AB; Box 5117, S-200 71 Malmö (SE). (81) Designated States: AT (European patent), BE (Euro- pean patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (Euro- pean patent), IT (European patent), JP, LU (Euro- pean patent), NL (European patent), NO, SE (Euro- pean patent), US. Published <i>With international search report.</i>	
(54) Title: A METHOD OF SIMULTANEOUSLY PRODUCING A LARGE NUMBER OF Leu ¹⁷ -VIP-ANALOGS AND NEW Leu ¹⁷ -VIP-ANALOGS (57) Abstract <p>A method of simultaneously producing a large number of individual C-terminally extended analogs of Leu¹⁷-VIP is described. Said method comprises the steps of synthesizing two separate mixtures of equally long oligonucleotides coding for N-terminal and C-terminal portions of Leu¹⁷-VIP analogs, respectively, each having a few defined triplets which are made ambiguous and a few identical bases at the 3'-end, mixing said two mixtures to form a mixture of partially double stranded equally long DNA sequences, which are enzymatically converted into double stranded DNA sequences, subjecting the last mentioned mixture to cleavage, with two different enzymes to produce DNA sequences, which are inserted into similarly cleaved vectors by ligation, whereupon the vectors, are transformed into hosts, propagating said hosts to form colonies, which are analyzed one by one to establish the DNA sequences which code for a single protein, propagating separately under expression conditions those hosts which contain vectors having identified protein coding sequences, whereupon the separately expressed proteins are cleaved to release the C-terminally extended analogs of Leu¹⁷-VIP. Additionally there are disclosed new C-terminally extended analogs of Leu¹⁷-VIP and plasmids containing genes coding for the new analogs of Leu¹⁷-VIP.</p>		

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A method of simultaneously producing a large number of Leu¹⁷-VIP-analogs and new Leu¹⁷-VIP-analogs.

The present invention relates to a method of simultaneously producing a large number of peptide analogs and to new peptide analogs. The invention relates specifically to a method of simultaneously producing a group of individual C-terminally extended analogs of Leu¹⁷-VIP (vasoactive intestinal polypeptide), to new C-terminally extended analogs of Leu¹⁷-VIP and to plasmids containing genes having DNA sequences which code for said new Leu¹⁷-VIP analogs.

10 Background

The pharmaceutical industry, and independent research workers, are constantly searching for new compounds, which slightly differ from useful known compounds, with the aim of finding more potent, more specific etc derivatives. In case the known compound of interest is a peptide or polypeptide, one or several amino acid residues of the amino acid sequence thereof are substituted for other natural or unnatural amino acid residues.

20 For screening purposes it would be desirable to be able to simultaneously produce a large number of analogs of a chosen peptide in order to save both time and money.

The present invention provides a method of simultaneously producing a large number of C-terminally extended analogs of Leu¹⁷-VIP, wherein all amino acid residues are of L-configuration.

Prior Art

30 Vasoactive intestinal polypeptide (VIP) is a highly basic 28 amino acids long peptide with a C-terminal amide belonging to the glucagon-secretin family. VIP was first isolated by S. Said and V. Mutt in 1970 (Said, S.I. and Mutt, V. (1970) Science 169, 1217-1218)

from porcine upper intestinal tissue, but has later been found both in nervous tissue and in endocrine cells (Said, S.I. (1982) Vasoactive Intestinal Peptide (Raven Press, N.Y.) and (Said, S.I. (1984) Peptides 5, 143-150). The biological effects of VIP include vasodilation of cerebral blood vessels, stimulation of prolactin release, stimulation of pancreatic exocrine secretion, effect on penile erection (Said, S.I. (1982) Vasoactive Intestinal Peptide (Raven Press, N.Y.) and (Rostène, W.H. (1984) Progress in Neurobiology 22, 103-129) and Mutt, V. (1983) in Brain Peptides, eds. Krieger, D.T., et al), potentiation of cholinergically stimulated salivary flow (Lundberg, J.M., et al (1982) Acta Physiol. Scand. 114, 329-337) and acting as surfactant in the lung (Barnes, P.J. (1987) TIPS, 8, 24-27).

Recently the precursor gene coding for human VIP has been isolated and sequenced (Bodner, M., et al. (1985) Proc. Natl. Acad. Sci. USA, 82, 3548-3551) and (Linder, S., et al. (1987) Proc. Natl. Acad. Sci. USA, 84, 605-609). The sequence data show that at the 3'-end of the gene coding for VIP there are three additional triplets coding for the amino acid sequence Gly-Lys-Arg. It has been shown that C-terminal Gly-Lys-Arg or Gly alone may act as substrate sites for C-terminal amidation by peptidylglycine α -amidating monooxygenase (PAMase) detected in various tissues (Bradbury, A.F., et al. (1982) Nature 298, 686-688) and (Eipper, B.A., et al (1983) Peptides 4, 921-928) and (Glembotski, C.C., et. al (1984) J. Biol. Chem. 259, 6385-6392) and (Gomez, S., et. al (1984) FEBS Lett. 167, 160-164) and (Eipper, B.A., et. al. (1985) Endocrinology, 116, 2497-2504) and Ouafik, H., et. al. (1987) Proc. Natl. Acad. Sci. USA, 84, 261-264).

It is obvious that large scale production by genetic engineering methods of VIP, a peptide hormone of broad biological activity, and analogs thereof,

would be desirable. Although the human precursor gene for VIP has been isolated and characterized (Bodner, M., et. al. (1985) Proc. Natl. Acad. Sci. USA, 82, 3548-3551) and Linder, S., et. al. (1987) Proc. Natl. Acad. Sci. USA, 84, 605-609), its expression in another
 5 organism has not been reported yet.

The C-terminally extended Leu¹⁷-VIP analogs of the present invention have been produced in E. coli.

Description of the invention

10 In one aspect of the invention there is provided a method of simultaneously producing a group of individual C-terminally extended analogs of Leu¹⁷-VIP (vasoactive intestinal polypeptide), said group consisting of equally long peptides having the amino acid sequence

15 His-Ser-Asp-Ala-Val-Phe-X-Asp-Asn-Tyr-Thr-Arg-Leu-Y-
 1 2 3 4 5 6 7 8 9 10 11 12 13 14

-Lys-Gln-Leu-Ala-Val-Z-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-
 20 15 16 17 18 19 20 21 22 23 24 25 26 27

-Asn-W
 28 29

25 wherein X represents Thr, Ser, Pro or Ala,
 Y represents Thr, Lys, Ile or Ala
 Z represents Thr, Lys, Ile or Arg, and
 W represents Gly or Gly-Lys-Arg,

30 and of equally long peptides which are also Leu¹⁷-VIP-
 -W²⁹ analogs and which arise from mutations when carrying out the method. Said method comprises the steps of
 simultaneously synthesizing a first mixture of
 35 equally long oligonucleotides which during synthesis
 are made ambiguous at the triplets coding for the
 amino acid residues at positions 7 and 14 of Leu¹⁷-VIP,
 and which have a few identical bases at the 3'-end,

simultaneously synthesizing a second mixture of equally long oligonucleotides which during synthesis are made ambiguous at the triplet coding for the amino acid residue at position 20 of Leu¹⁷-VIP, and which
5 have a few identical bases at the 3'-end, these being complementary to the few bases at the 3'-end of the oligonucleotides in the first mixture,

mixing said first mixture with said second mixture, whereby the few bases at the 3'-end of the oligonucleo-
10 tides of the first mixture anneal to the few bases at the 3'-end of the oligonucleotides of the second mixture, resulting in a mixture of partially double stranded equally long DNA sequences, which thereafter are enzymatically converted into fully double stranded
15 DNA sequences,

subjecting said mixture of double stranded DNA sequences, which include at the 3'-end a site for cleavage with a first enzyme and at the 5'-end a site for cleavage with a second enzyme, to cleavage, simul-
20 taneously or consecutively, with the first and the second enzyme to produce DNA sequences with 3'- and 5'-ends which are ligatable to the 5'- and 3'-ends of similarly cleaved vectors,

inserting the thus cleaved DNA sequences into
25 the thus cleaved vectors by ligation to produce a mixture of vectors, which are then transformed into hosts in per se known manner,

propagating said hosts to form colonies, which are analyzed one by one to establish those DNA sequences
30 which code for a single protein,

propagating separately under expression conditions those hosts which contain vectors having identified protein coding sequences, the C-terminal portions of which correspond to an individual Leu¹⁷-VIP-W²⁹
35 analog, whereupon the separately expressed proteins are cleaved to release the C-terminal Leu¹⁷-VIP-W²⁹

5

analogues forming a group of said individual equally long peptides.

In one embodiment of this aspect of the invention, wherein W represents Gly, the Leu¹⁷-VIP-W²⁹ analogues which arise from mutations when carrying out the method are

Asp¹-Ala⁷-Ile¹⁴-Thr²⁰-VIP-Gly²⁹
 Tyr¹-Pro⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 10 Tyr²-Thr⁷-Tyr²-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 Gly³-Thr⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁴-Thr⁷-Arg¹⁴-Thr²⁰-VIP-Gly²⁹
 Phe⁵-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Asn⁸-Arg¹⁴-Arg²⁰-VIP-Gly²⁹
 15 Pro⁷-Gly⁸-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Gln⁸-Ile¹⁴-Phe¹⁹-Arg²⁰-VIP-Gly²⁹
 Ser⁷-Pro⁸-Ile¹⁴-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Pro¹¹-Lys¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Pro¹²-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 20 Thr⁷-Lys¹⁴-Asn¹⁵-Ile²⁰-VIP-Gly²⁹
 Pro⁷-Ile¹⁴-Arg¹⁵-Lys²⁰-VIP-Gly²⁹
 Ala⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Ile¹⁴-Thr¹⁵-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-His¹⁶-Lys²⁰-VIP-Gly²⁹
 25 Pro⁷-Ile¹⁴-His¹⁶-Thr²⁰-VIP-Gly²⁹
 Thr⁷-Ile¹⁴-Phe¹⁹-Ile²⁰-VIP-Gly²⁹
 Pro⁷-Thr¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹
 Ser⁷-Lys¹⁴-Thr²⁰-Ile²⁴-VIP-Gly²⁹
 Thr⁷-Thr¹⁴-Lys²⁰-Asp²⁸-VIP-Gly²⁹
 30 Gly⁷-Lys¹⁴-Lys²⁰-VIP-Gly²⁹, and
 Ala⁷-Asn¹⁴-Arg²⁰-VIP-Gly²⁹.

In another embodiment of this aspect of the invention, wherein W represents Gly-Lys-Arg, the Leu¹⁷-VIP-W²⁹ analogues which arise from mutations when carrying out the method are

6

Phe²-Pro⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Val³-Thr⁷-Lys¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁴-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Phe⁵-Pro⁷-Thr¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 5 Thr⁶-Ala⁷-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Val⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Ile¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Arg¹³-Lys¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 10 Pro⁷-Gln¹³-Lys¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Arg¹³-Thr¹⁴-Gln¹⁵-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 15 Ser⁷-Lys¹⁴-Asn¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Asp¹⁹-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Gly¹⁹-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Thr¹⁴-Ile²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ala⁷-Ile¹⁴-Arg²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 20 Ser⁷-Ile¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Ile¹⁴-Thr²⁰-Tyr²⁵-VIP-Gly²⁹-Lys³⁰-Arg³¹ and
 Ser⁷-Thr¹⁴-Ile²⁰-Val²⁶-VIP-Gly²⁹-Lys³⁰-Arg³¹.

Examples of hosts to be used in the above method
 are bacteria, e.g. of genus *Bacillus* or *Escherichia*
 25 *coli*, and yeasts, e.g. *Saccharomyces cerevisiae*.
 Examples of vectors to be used in the above method
 are bacteriophages and plasmids.

In a preferred embodiment of this aspect of the
 invention, the vectors are plasmids and the hosts
 30 are *E. coli*.

As a result of carrying out said preferred embodi-
 ment of the method of the invention there was produced
 a large number of C-terminally extended Leu¹⁷-VIP
 analogs, which form an additional aspect of the in-
 35 vention.

7

Thus, in an additional aspect of the invention there is provided a C-terminally extended analog of Leu¹⁷-VIP which is chosen from the group consisting of peptides having the amino acid sequence

5 His-Ser-Asp-Ala-Val-Phe-X-Asp-Asn-Tyr-Thr-Arg-Leu-Y-
 1 2 3 4 5 6 7 8 9 10 11 12 13 14
 -Lys-Gln-Leu-Ala-Val-Z-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-
 10 15 16 17 18 19 20 21 22 23 24 25 26 27
 -Asn-W
 28 29

15 wherein X represents Thr, Ser, Pro or Ala,
 Y represents Thr, Lys, Ile or Ala
 Z represents Thr, Lys, Ile or Arg, and
 W represents Gly or Gly-Lys-Arg,
 (which resulted from the method and which were ori-
 20 ginally planned to be produced)
 and

analogs of Leu¹⁷-VIP having the same amino acid sequence wherein W represents Gly,

except for His in position 1 which is substituted
 25 by Asp when X = Ala, Y = Ile and Z = Thr,
 except for His in position 1 which is substituted
 by Tyr when X = Pro, Y = Thr and Z = Lys,
 except for Ser in position 2 which is substituted
 by Tyr and Asp in position 8 which is substituted
 30 by Tyr when X = Thr, Y = Thr and Z = Thr,
 except for Asp in position 3 which is substituted
 by Gly when X = Thr, Y = Thr and Z = Arg,
 except for Ala in position 4 which is substituted
 by Pro when X = Thr, Y = Arg and Z = Thr,
 35 except for Val in position 5 which is substituted
 by Phe when X = Thr, Y = Thr and Z = Lys,

8

except for Asp in position 8 which is substituted
by Asn when X = Thr, Y = Arg and Z = Arg,
except for Asp in position 8 which is substituted
by Gly when X = Pro, Y = Thr and X = Lys,
5 except for Asp in position 8 which is substituted
by Gln and Val in position 19 which is substituted
by Phe when X = Pro, Y = Ile and Z = Arg,
except for Asp in position 8 which is substituted
by Pro when X = Ser, Y = Ile and Z = Arg,
10 except for Thr in position 11 which is substituted
by Pro when X = Ala, Y = Lys and Z = Lys,
except for Arg in position 12 which is substituted
by Pro when X = Pro, Y = Thr and Z = Thr,
except for Lys in position 15 which is substituted
15 by Asn when X = Thr, Y = Lys and Z = Ile,
except for Lys in position 15 which is substituted
by Arg when X = Pro, Y = Ile and Z = Lys,
except for Lys in position 15 which is substituted
by Gln when X = Ala, Y = Lys and Z = Arg,
20 except for Lys in position 15 which is substituted
by Thr when X = Ala, Y = Ile and Z = Lys,
except for Gln in position 16 which is substituted
by His when X = Thr, Y = Lys and Z = Lys,
except for Gln in position 16 which is substituted
25 by His when X = Pro, Y = Ile and Z = Thr,
except for Val in position 19 which is substituted
by Phe when X = Thr, Y = Ile and Z = Ile,
except for Asn in position 24 which is substituted
by Lys when X = Pro, Y = Thr and Z = Thr,
30 except for Asn in position 24 which is substituted
by Ile when X = Ser, Y = Lys and Z = Thr,
except for Asn in position 28 which is substituted
by Asp when X = Thr, Y = Arg and Z = Lys,
and X = Gly when Y = Lys and Z = Lys, and
35 X = Ala when Y = Asn and Z = Arg,
[these analogs can be named with regard to VIP as
follows:

9

Asp¹-Ala⁷-Ile¹⁴-Thr²⁰-VIP-Gly²⁹
 Tyr¹-Pro⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Tyr²-Thr⁷-Tyr⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 Gly³-Thr⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹
 5 Pro⁴-Thr⁷-Arg¹⁴-Thr²⁰-VIP-Gly²⁹
 Phe⁵-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Asn⁸-Arg¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁷-Gly⁸-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Gln⁸-Ile¹⁴-Phe¹⁹-Arg²⁰-VIP-Gly²⁹
 10 Ser⁷-Pro⁸-Ile¹⁴-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Pro¹¹-Lys¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Pro¹²-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-Asn¹⁵-Ile²⁰-VIP-Gly²⁹
 Pro⁷-Ile¹⁴-Arg¹⁵-Lys²⁰-VIP-Gly²⁹
 15 Ala⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Ile¹⁴-Thr¹⁵-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-His¹⁶-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Ile¹⁴-His¹⁶-Thr²⁰-VIP-Gly²⁹
 Thr⁷-Ile¹⁴-Phe¹⁹-Ile²⁰-VIP-Gly²⁹
 20 Pro⁷-Thr¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹
 Ser⁷-Lys¹⁴-Thr²⁰-Ile²⁴-VIP-Gly²⁹
 Thr⁷-Thr¹⁴-Lys²⁰-Asp²⁸-VIP-Gly²⁹
 Gly⁷-Lys¹⁴-Lys²⁰-VIP-Gly²⁹ and
 Ala⁷-Asn¹⁴-Arg²⁰-VIP-Gly²⁹]

25 (which arose as mutations when carrying out one run
 of the preferred embodiment of the method of the in-
 vention)

and analogs of Leu¹⁷-VIP having the same amino acid
 30 sequence wherein W represents -Gly-Lys-Arg
 except for Ser in position 2 which is substituted
 by Phe when X = Pro, Y = Thr and X = Arg,
 except for Asp in position 3 which is substituted
 by Val when X = Thr, Y = Lys and Z = Ile,
 35 except for Ala in position 4 which is substituted
 by Pro when X = Thr, Y = Thr and Z = Lys,

10

except for Val in position 5 which is substituted
by Phe when X = Pro, Y = Thr and Z = Ile,
except for Phe in position 6 which is substituted
by Thr when X = Ala, Y = Thr and Z = Thr,
5 except for Asp in position 8 which is substituted
by Val when X = Thr, Y = Thr and Z = Thr,
except for Thr in position 11 which is substituted
by Pro when X = Pro, Y = Thr and Z = Lys,
10 except for Thr in position 11 which is substituted
by Pro when X = Pro, Y = Ile and Z = Arg,
except for Leu in position 13 which is substituted
by Arg when X = Thr, Y = Lys and Z = Arg,
except for Leu in position 13 which is substituted
15 by Gln when X = Pro, Y = Lys and Z = Lys,
except for Leu in position 13 which is substituted
by Pro when X = Pro, Y = Thr and Z = Thr,
except for Leu in position 13 which is substituted
by Pro when X = Pro, Y = Thr and Z = Arg,
20 except for Leu in position 13 which is substituted
by Arg and Lys in position 15 which is substituted
by Gln when X = Ser, Y = Thr and Z = Thr,
except for Lys in position 15 which is substituted
by Gln when X = Pro, Y = Lys and Z = Arg,
25 except for Lys in position 15 which is substituted
by Asn when X = Ser, Y = Lys and Z = Arg,
except for Val in position 19 which is substituted
by Asp when X = Thr, Y = Lys and Z = Lys,
except for Val in position 19 which is substituted
30 by Gly when X = Thr, Y = Lys and Z = Thr,
except for Asn in position 24 which is substituted
by Asp when X = Thr, Y = Thr and Z = Ile,
except for Asn in position 24 which is substituted
by Asp when X = Ala, Y = Ile and Z = Arg,
35 except for Asn in position 24 which is substituted
by Lys when X = Ser, Y = Ile and Z = Thr,
except for Ser in position 25 which is substituted
by Tyr when X = Pro, Y = Ile and Z = Thr, and

except for Ile in position 26 which is substituted by Val when X = Ser, Y = Thr and Z = Ile, [these analogs can be named with regard to VIP as follows:

5 Phe²-Pro⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Val³-Thr⁷-Lys¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁴-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Phe⁵-Pro⁷-Thr¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 10 Thr⁶-Ala⁷-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Val⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Ile¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Arg¹³-Lys¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 15 Pro⁷-Gln¹³-Lys¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Arg¹³-Thr¹⁴-Gln¹⁵-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 20 Ser⁷-Lys¹⁴-Asn¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Asp¹⁹-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Gly¹⁹-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Thr¹⁴-Ile²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ala⁷-Ile¹⁴-Arg²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 25 Ser⁷-Ile¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Ile¹⁴-Thr²⁰-Tyr²⁵-VIP-Gly²⁹-Lys³⁰-Arg³¹ and
 Ser⁷-Thr¹⁴-Ile²⁰-Val²⁶-VIP-Gly²⁹-Lys³⁰-Arg³¹]

(which arose as mutations when carrying out a second run of the preferred embodiment of the method of the invention).

All the amino acid residues in the C-terminally extended Leu¹⁷-VIP analogs of the invention are of L-configuration.

35 In a further aspect of the invention there is provided a plasmid containing a gene having a DNA

12

sequence which codes for a C-terminally extended Leu¹⁷-VIP analog of the invention.

5 All the C-terminally extended Leu¹⁷-VIP analogs of the invention have, besides similar properties to natural human VIP, some useful properties in common, namely, they are ligands at VIP receptors, they are resistant to oxidation and can be kept at acidic pH in solution (due to the replacement of methionine in position 17 of natural VIP by leucine), and, since
10 they are C-terminally extended, they exhibit a prolonged effect both in vitro and in vivo, which is considered to be of advantage in view of the extremely short biological half-life of VIP.

Thus the C-terminally extended Leu¹⁷-VIP analogs
15 of the invention are useful as potential active ingredients in new pharmaceuticals, as well as in cosmetics (for hydration of the skin).

The new pharmaceuticals are intended to be administered

- 20 1) for the treatment of asthma and of constriction of the upper airways in general, specially for vasodilation in the lungs,
- 2) as pheripheric vasodilators to be locally applied for i.a. erection of penis,
- 25 3) for the treatment of VIPOMA (a VIP producing intestinal tumour which causes death of a human by loss of 5-15 liters of water per day),
- 4) for the treatment of disorders of blood flow, blood-pressure, intestinal motility and urinary
30 bladder,
- 5) for potentiation of salivation e.g. for the treatment of blocked or reduced salivation (e.g. due to administration of antipsychotic drugs or anti-depressants), and
- 35 6) for the stimulation of pancreatic juice secretion.

The composition of pharmaceuticals including the active ingredient will depend on the intended

route of administration and the specific disorder to be treated. A nasal or oral spray is suitable when asthma is to be treated. Aqueous solutions or tablets for oral administration will be suitable for other indications. In all types of pharmaceuticals the active ingredient is in admixture with pharmaceutically acceptable excipients and/or diluents normally used in the field of pharmacy.

General strategy to obtain Leu¹⁷-VIP analog-coding

10 DNA sequences cloned in an E. coli expression vector

A large number of artificial genes coding for C-terminally extended Leu¹⁷-VIP analogs were planned to be obtained directly as parts of an E. coli plasmid expression vector by performing only one cloning step.

15 The synthetic strategy utilized the principle of a method described originally [Simoncsits, A., Kálmán, M., Cserpán, J. and Kari, C. (1984) Nucleic Acids Res. Symp. Ser. 14, 321] for obtaining a large number of artificial E. coli promoter derivatives. Briefly,

20 two single-stranded oligodeoxyribonucleotides were chemically synthesized and were annealed [Itakura, K. (1982) TIBS, 7, 442] through their 3'-terminal complementary sequences. The partial duplex obtained so covered the whole Leu¹⁷-VIP coding region and contained

25 additional terminal sequences to be used for cloning. The two oligodeoxyribonucleotides were obtained by ambiguous chemical synthesis so that the mixture of all four activated nucleotide monomers were used at certain predetermined positions (i.e. in triplets

30 coding for the amino acid residues 7, 14 and 20 of Leu¹⁷-VIP) which were outside of the annealing region. The annealed partial duplex was converted by a mutually primed synthesis effected by DNA polymerase (Klenow fragment) into double-stranded DNA mixture containing

35 all possible homoduplexes. These were then cleaved with two different restriction endonucleases the cleavage sites of them being located upstream and downstream

of the Leu¹⁷-VIP coding region and the cleaved mixture was ligated with the appropriately cleaved expression vector pPEX. The ligated mixture was transformed into competent *E. coli* cells and the recombinants containing
5 Leu¹⁷-VIP related coding sequences were selected by colony hybridization. The positive recombinants were further analyzed by nucleotide sequencing to identify the particular mutations obtained in the cloned Leu¹⁷-VIP coding region.

10 During the design of the synthetic oligodeoxy-ribonucleotides, it was considered that they should code for a methionine preceding the Leu¹⁷-VIP coding region and for a Gly (VIPa analogs) or a Gly-Lys-Arg (VIPb-analogs) extension at the carboxy terminus.
15 Methionine was included to be able to release the Leu¹⁷-VIP analogs from the fusion protein by CNBr cleavage, while the carboxy terminal extensions could serve as substrate sites for amidation catalysed by peptidylglycine α -amidating monooxygenase (PAMase)
20 [Bradburg, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) *Nature* 298, 686]. The positions of the mixed chemical couplings within the nucleotide sequences were chosen to be located in the predetermined X, Y and Z codons so that after performing the steps shown in Scheme 1,
25 the maximum number of amino acid variations be obtained for X, Y and Z. By choosing the first (for codon X) or the second (for codons Y and Z) base of the target codons as mixed positions, four amino acids could be obtained of each three codon variations, yielding
30 altogether 64 possible Leu¹⁷-VIP analogs after expression.

The VIP1 oligonucleotide, shown as the upper-strand of the partial, annealed duplex in Scheme 1 contains two mixed positions (N), while the lower
35 strand VIP2 oligonucleotide contains only one. The VIP2 oligonucleotide was prepared in two variations

carrying information for C-terminal extensions as above (VIP2a and VIP2b oligonucleotides in Scheme 1). To obtain both VIPa and VIPb analogs, two separate experiments were carried out using VIP1 + VIP2a or
5 VIP1 + VIP2b oligonucleotides, respectively.

The double stranded DNA mixture obtained by Klenow polymerase reaction contained homoduplex molecules which were separated by cloning into an expression vector. In principle, all recombinants should contain
10 only one type of mutant gene coding for a particular Leu¹⁷-VIP analog.

pPEX vector

pPEX is a highly efficient E. coli expression vector constructed from a rac fusion [Boros, J.,
15 Lukacsovich, T., Baliko, G. and Venetianer, P. (1986) Gene 42, 97] promoter vector La int 23 named as pPEX by us. The La int 23 vector, which expresses under rac promoter control a fusion protein composed of 280 amino acids of E. coli β -galactosidase and part
20 of the bacterial CAT (chloramphenicol acetyl transferase) was modified by eliminating its unique BamHI site and by replacing its CAT coding region located between ClaI and EcoRI sites with a synthetic polycloning region. The relevant region of the pPEX vector
25 obtained so is shown in Scheme 2. The vector contains unique BamHI, KpnI, SacI, ApaI and EcoRI sites in the polycloning region and β -galactosidase gene fusions can be performed using the BamHI and any of the other unique sites. When the Leu¹⁷-VIP coding genes are
30 cloned into the BamHI and EcoRI sites of the pPEX vector, the resulting gene fusions code for 314 and 316 amino acids long β -galactosidase-VIPa and β -galactosidase-VIPb fusion proteins, respectively, of which proteins 284 amino acids are derived from the fusion
35 partner (Scheme 1).

EXAMPLEPreparation of the mixture of DNA regions coding for
Leu¹⁷-VIP analogsMATERIALS

5 Restriction enzymes and T4 DNA ligase were obtained
from New England Biolabs. T4 polynucleotide kinase
and Klenow polymerase were from Boehringer, [γ -³²P]ATP
(>5000 Ci/mmol) and [α -³²P]dATP (800 Ci/mmol) were
from Amersham, Rabbit antisera directed against BSA-VIP
10 conjugates was provided by Dr. Per Askelöf (SBL, Stock-
holm, Sweden). Immunoblot assay kit containing anti-
-rabbit IgG-alkaline phosphatase conjugate and color
development reagents BCIP/NBT was purchased from Bio-Rad.
T₁ RNase was purchased from Calbiochem.

15 Oligodeoxyribonucleotides

CCGGATCCATATGCACTCTGACGCTGTTTTTCNCTGACAACTACACT-
CGTCTGANAAAACAGCTGGCT (VIP1 oligonucleotide),

AAGAATTCAGCCGTTTCAGGATAGAGTTCAGGTACTTTNTAACAGCC-
AGCTGT (VIP2a oligonucleotide),

20 AAGAATTCACGTTTGCCGTTTCAGGATAGAGTTCAGGTACTTTNTA-
ACAGCCAGCTGT (VIP2b oligonucleotide) and

CAGGGTGAAACGCAGGTCGCCAGCGGC (lac Z 27-mer primer)
oligonucleotides were prepared on an automatic DNA
synthesizer by the phosphoramidite chemistry (Pharmacia
25 Gene Assembler) and were purified by electrophoresis
on polyacrylamide gels containing 8 M urea. Oligonucleo-
tides were 5'-phosphorylated with [γ -³²P]ATP by using
T4 polynucleotide kinase [Maniatis, T., Fritsch, E.F.
and Sambrook, J. (1982) Molecular Cloning: A labora-
30 tory manual, Cold Spring Harbor Laboratory, Cold Spring
Harbor N.Y.] when they were to be used as either hybrid-
ization probe or sequencing primer.

100 pmol of VIP1 oligonucleotide was mixed with
100 pmole of either VIP2a or VIP2b oligonucleotide
35 in 50 μ l of water, the mixture was kept at 90°C for
3 min and was let to cool to room temperature in approx.

1 hr period. The solution was made up to 100 μ l containing 10 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 50 μ M each of dCTP, dGTP and TTP, 5 μ l of [α - ^{32}P]dATP (approx. 62.5 pmol) and 1 μ l of 5U/ μ l Klenow polymerase. After
5 15 min 10 μ l of 1 mM dNTP mixture (containing all four deoxynucleoside 5'-triphosphates) was added and the solution was incubated at room temperature for 15 min. The DNA was precipitated with the help of 1 μ l of 10 μ g/ μ l yeast carrier tRNA (ctRNA) by ethanol
10 precipitation and purified by 10% acrylamide-8 M urea gel electrophoresis at 400V for 2 hrs (gel thickness 0.4 mm). The gel was radioautographed, the major radioactive band was cut out and soaked in 300 μ l of 50 mM NaCl - 0.1% SDS solution at 37°C for 12 hrs. The super-
15 natant was phenol extracted and the precipitation was carried out by adding 1 μ l of 10 μ g/ μ l ctRNA, 30 μ l of 3 M NaOAc, pH 5.2 and 900 μ l of ethanol in a liquid nitrogen bath. Centrifugation (14 000 rpm, 3 min) resulted in a radioactive pellet which was
20 washed with ethanol, dried and dissolved in sterile water.

20 pmol of the radioactive Leu^{17} -VIP-DNA obtained as above was treated with 200 units of BamHI and EcoRI each in 200 μ l of 100 mM NaCl, 50 mM Tris HCl, pH
25 7.5, 10 mM $MgCl_2$, 1 mM DTT (high-salt buffer) at 37°C for 20 hrs. The reaction mixture was heated to 60°C for 10 min, phenol extracted (two times), and the DNA was recovered from the waterphase by ethanol precipitation as above (two times) followed by ethanol
30 washing, drying and redissolving in sterile water. Analysis of the mixture by 10% acrylamide-8 M urea electrophoresis showed that only approx. 50% of the duplex was cleaved and the fraction of the DNA which had been cleaved by both enzymes was only approx.
35 30%. Nevertheless, the mixture was used as it was for cloning into BamHI-EcoRI cleaved pPEX vector.

Cleavage of pPEX vector with BamHI and EcoRI

2 µg of pPEX 13 was treated with 10-10 units of BamHI and EcoRI in 20 µl of high salt buffer at 37°C for 4 hrs. The reaction mixture was applied onto
5 an agarose gel (0.5%) and the electrophoresis was performed in TAE buffer (40 mM Tris-acetate, 2 mM EDTA pH 7.5) at 60V for 1 hr in the presence of ethidium bromide. The linear vector band was cut out, electroeluted, phenol extracted twice, ethanol precipitated,
10 washed with ethanol and dried. The pellet was dissolved in sterile water.

Cloning of Leu¹⁷-VIP-DNA into pPEX vector

0.2 µg BamHI-EcoRI cleaved pPEX vector and approx. 5 pmol BamHI-EcoRI Leu¹⁷-VIP-DNA obtained as above
15 were reacted in 20 µl reaction mixture containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP and 80 units of T4 DNA ligase at 15°C for 12 hrs. The reaction mixture was transformed into JM101 E. coli cells [genotype (supE, thi, Δ(lac-proAB), [F',
20 traD36, proAB, lacI^qΔM15), Messing, J., Crea, R. and Seeburg, P.H. (1981) Nucleic Acids Res. 9, 309-321] using a frozen stock [Hanahan, D. in DNA cloning, Vol I., edited by Glover, D.M. IRC Press Limited (1985), pp 109-135, protocol 3]. Ampicillin resistant colonies
25 were picked for colony hybridization [Grunstein, M. and Hogness, D.S. (1975) Proc. Natl. Acad. Sci. USA 72, 3961-3965] with ³²P-labeled VIP1 oligonucleotide probe. Approx. 80-90% of the colonies were positive in this test for both VIPa and VIPb mutants. 120 colonies
30 of both series were taken for plasmid preparation and sequencing.

Plasmid preparation and sequencing

A single colony was inoculated into 3 ml of LB-medium containing 100 µg/ml ampicillin and the culture
35 was shaken for 12-16 hrs at 37°C. Plasmid DNA was prepared by a rapid alkaline extraction procedure [Birnboim, H.C. and Doly, J. (1979) Nucleic Acids

Res., 7, 1513-1523]. The plasmid DNA was further treated with 5 units of T_1 RNase in 100 μ l solution containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA at 37°C for 30 min followed by phenol extraction and ethanol precipitation.

5 The pellet was taken up in 30 μ l of sterile water.

Dideoxynucleotide sequencing was performed on HindIII linearized plasmid template (0.2-0.4 μ g) using 5'- 32 P-phosphorylated lac Z 27-mer sequencing primer (0.25 pmol) and heat denaturation as described [Hong, G.F., (1982) Bioscience Reports 2, 907]. Sequencing reactions for 24 clones were performed at the same time.

Expression of Leu¹⁷-VIP analogs

E. coli JM101 cells transformed with pPEX Leu¹⁷-VIP analog plasmids were grown at 37°C, shaking 250 rpm, in 1 ml LB medium containing 0.1 mg/ml ampicillin. When the cell density reached 0.5 at A=600 nm the cells were induced either by addition of IPTG (final concentration 2.5 mM) and shaken at 250 rpm for 4 hours at 37°C or by addition of lactose (final concentration 1%) and shaken at 250 rpm for 20 hours. After induction the cells were collected by centrifugation at 12 000 rpm in an Eppendorf centrifuge for 1 minute, lysed at 100°C for 3 minutes in 300 μ l of lysis buffer (0.125 M Tris-HCl pH 6.8, 30% glycerol, 2% SDS, 6 M urea and 1 M 2-mercaptoethanol) and 30 μ l of non-induced extracts and 30 μ l of induced extracts were run on SDS-10% polyacrylamide gels according to Laemmli, U.K. (1970) Nature, 227, 680-685 to monitor the degree of protein expression as shown by coomassie brilliant blue staining of the gels. As estimated from the gels the amount of expressed fusion protein was more than 60% of total cell proteins.

Immunological recognition/detection of β -galactosidase-Leu¹⁷-VIP analogs

6 μ l aliquot of non-induced and 6 μ l of 50x diluted induced samples were run on SDS-10% polyacrylamide

gels in parallel and half of the gels were stained
with coomassie brilliant blue and the other half of
the gels were electroblotted onto nitrocellulose papers.
The blotted nitrocellulose papers were incubated with
5 a polyclonal rabbit serum containing antibodies directed
against BSA-VIP, and the immunoblot was developed
by treatments with goat anti rabbit IgG conjugated
alkaline phosphatase and BCIP/NBT color development
solution (Bio-Rad immuno assay kit) according to the
10 manufacturer's instructions. A very strong immuno-
staining was obtained with the fusion proteins con-
taining Leu¹⁷-VIP analogs.

1 liter scale purification of expressed β -galactosidase -
-Leu¹⁷-VIP analog fusion proteins

15 E. coli JM101 cells carrying the pPEX-Leu¹⁷-VIP
analog plasmids were shaken (250 rpm) in 1 liter LB
medium containing 0.1 mg/ml ampicillin at 37°C. When
the absorbance at 600 nm reached 0.5 the cells were
induced with lactose (1% final concentration) and
20 shaken at 250 rpm at 37°C for 20 hours. After induction
the cells were harvested by centrifugation at 2000 x
g, 4°C for 10 minutes, washed with 40 ml of TBS, pH
7.6, and resuspended in 30 ml TBS containing 10 μ M
PMSF and 0.5 mM EDTA. The cell suspension was then
25 freeze-thawed and sonicated (five 1 minute bursts)
in a Branson sonifier, then centrifuged at 17000 x g,
4°C, for 30 minutes. The pellet containing the fusion
protein was homogenized in 50 ml 6 M guanidium chloride
containing 1% 2-mercaptoethanol according to Goeddel,
30 D.V. et. al. (1979) PNAS (USA), 76, 106-110 and centri-
fuged at 60 000 x g for 60 minutes. The clear, amber
supernatant containing the fusion protein was diluted
to 100 ml with 6 M guanidium chloride - 1% 2-mercapto-
ethanol and 172 ml TBS was added dropwise with vigorous
35 stirring which rendered most of the fusion protein
to precipitate while most of the host proteins remained
in solution. The slurry was stirred at room temperatur

21

for 30 minutes before centrifugation at 10 000 x g for 30 minutes at 10°C. The pellet so obtained contains most of the β -galactosidase-Leu¹⁷-VIP analog fusion protein.

5 CNBr cleavage of fusion protein

The partially purified β -galactosidase - Leu¹⁷-VIP analog fusion protein was dissolved by homogenization in 25 ml 70% HCOOH and 1.5 g CNBr was added per gram precipitate. The solution was stirred at room temperature for 70 hours and then evaporated at room temperature under reduced pressure using a Rotavapor. 20 ml 50 mM HCl in methanol was added per gram fusion protein to the residue and stirred at room temperature over night. Then the slurry was centrifuged at 2000 x g, 4°C, for 10 minutes and the supernatant collected.

15 Purification of Leu¹⁷-VIP analogs

The supernatant containing the Leu¹⁷-VIP analog was either 1)

- freeze-dried and the Leu¹⁷-VIP analog-containing residue was dissolved in 1% TFA and purified on HPLC (C₁₈-Bondapack, reverse phase)

or 2) subjected to further purification as follows before the HPLC step.

- To the acidic methanol phase containing the Leu¹⁷-VIP analog solid NaCl (0.2 g/ml) and 4 volumes of ice cold diethylether were added.

The precipitate (containing Leu¹⁷-VIP analog) was collected on a sintered glass funnel (pore size no 3) and dissolved by washing with 5 ml 1 M acetic acid. The dissolved Leu¹⁷-VIP analog was desalted with a Sephadex G25 column (7 cm long, 2 cm diameter) and freeze-dried before dissolution in 1% TFA and purified on HPLC with a 20%-40% acetonitrile linear gradient in 1% TFA. The Leu¹⁷-VIP analog was eluted at 33% acetonitrile. The purified Leu¹⁷-VIP analog was then tested in biochemical and biological assay systems.

EXAMPLE: VIPa (X=Thr, Y=Arg, Z=Lys, W=Gly)

The fusion protein β -galactosidase - VIPa was expressed in 1 liter LB medium containing 0.1 mg/ml ampicillin and purified as described above. The fusion
5 protein was cleaved with CNBr and VIPa so obtained was further purified as described above and tested in the following assay systems:

- 1) Radioimmunoassay (RIA)
- 10 2) Radioreceptor assay
- 3) Stimulation of 3',5'-cyclic AMP production
- 4) Bicarbonate secretion in cat in vivo

1) Radioimmunoassay (RIA)

- 15 In the VIP - RIA kit purchased from Peninsula Laboratories (USA) recombinant VIPa is recognized as VIP when measured according to the manufacturer's recommendation, based on the method of Fahrenkrug, J. and Schaffalitzky De Muckadell, O. (1977) J. Lab.
20 Clin. Med. 89, 1379-1388.

2) Radioreceptor assay

- Radioreceptor assay was carried out on membranes from rat cerebral cortex using chloramin-T iodinated ^{125}I -VIP (Halldén, G. et. al. (1986) Reg. Pep. 16,
25 183-188) at 0.5 nM concentration, studying the displacement of the labeled VIP by purified VIP from porcine intestine and by recombinant VIPa from E. coli as described by Abens, J. et al. (1984) Peptides 5, 375-377. The affinity of recombinant VIPa was within
30 the experimental error identical to that of VIP purified from porcine intestine with a Kd value of 1.5-1.8 nM at equilibrium, proving that recombinant VIPa is a high affinity ligand at VIP receptors, binding in a manner indistinguishable from that of VIP obtained
35 from other sources.

3) Stimulation of 3',5'-cyclic AMP synthesis

Stimulation of 3',5'-cyclic AMP (cAMP) synthesis in tissue slices from rat cerebral cortex by VIP purified from porcine and recombinant VIPa was studied to establish whether or not recombinant VIPa, a ligand of the VIP receptor according to the result in 2), behaves as an agonist or an antagonist. The experiments were carried out as follows:

Rat cerebral cortical slices (0.4 mm x 0.4 mm) were preincubated in Krebs Ringers bicarbonate buffer, bubbled with O₂/CO₂ (95%/5%) (V/V) for 60 minutes at 36°C in the presence of 10 mM theophylline (a phosphodiesterase inhibitor). The tissue slices were then incubated in 600 µl total volume with porcine VIP at 10, 50, 100 nM, 1 and 10 µM concentration for 10 minutes and with recombinant VIPa at 10, 50, 100 and 300 nM concentration (estimated by 1)) for 10 minutes. The incubations were terminated by addition of 150 µl EGTA (100 mM) solution followed by placing the test tubes into a boiling water bath for 3 minutes. The cAMP content was measured according to Brown, B.L. et al. (1972) Adv. Cycl. Nucl. Res. 2, 25-40. The results indicate that at 10 minutes incubation time recombinant VIPa produced an increase in cAMP levels and thus act as agonist at the VIP-receptor coupled adenylate cyclase, which is thought to mediate the physiological actions of VIP (cf. Rostène, W.H. (1984) Progr. Neurobiol. 22, 103-129).

4) Biological activity of VIPa

The biological activity of recombinant VIPa was also examined in the in vivo assay on stimulation of bicarbonate secretion from cat pancreas according to Mutt, V. and Söderberg, U. (1959) Arkiv Kem. 15, 63-68.

Recombinant VIPa (16 µg injected) was in this assay approximately 70% as efficient as porcine VIP.

24

Recombinant VIPb was, when tested at the same concentration in this assay, at least as efficient as porcine VIP.

VIPI oligonucleotide

N: A, C, G or T

Klenow polymerase + dNTP

BamHI

CCCGATCCATATGCACCTCTCAAGCTGTTTNDCTGCAACTACACTGCTCTGCANAAGCAAGCTGGCTGTTANAAGTAAGTACCTGACCTCTATCTCTGAC; EGGCTAGGTATACGTGACACTGCGCAAAAGCACTGTTCATGTGAGCAGACACNTTTTGTGCAAGCAATNTTTCATGACTTTCAGATAGGACCTTG

VIPa and VIPb duplexes

EcoRI

GGCTGAATTCCTT
DDGACTTAAGAA

EcoRI

GC¹AA²CG³TT⁴GA⁵TT⁶CT⁷
 CCG¹TT²GC³AA⁴CT⁵TA⁶CA⁷AA⁸

- 1, BamHI and EcoRI cleavage
- 2, Cloning into BamHI and EcoRI cleaved pPEX vector
- 3, Selection of individual mutants by nucleotide sequencing

cont.

SCHEME 1 (cont.)



β-galactosidase

IleAspSerTrpIleHisMetHisSerAspAlaIlePhe X AspAsnTyrThrArgLeu Y LysGlnLeuAlaVal Z LysTyrLeuAsnSerIleLeuAsn
ATCGATTCTTGGATCCATATGCACTCTGACGCTGTTTTCCTGCACTACACTGCTGCAAGAGCTGCTGTTTANAAGTACCTGAACCTCTATCTCTGAAC
TACCTAACAACCTAGGTATACGTGACACTGCGACAAAGAGCACTGTTGATGTGAGCAGACTTTTTTGTCGACGCAATTTTTCATGCTGCTGAGATAGGACTTG
ClaI BamHI

W Gly... a
GGCTGAATTC
CCGACTTAAG
EcoRI
W GlyLysArg...
GGCAAAAGCTTGAATTC
CCGTTTCCAACTTAAG
EcoRI b

X =	Y =	Z =
Thr	Arg	Lys
-ACT-	-AGA-	-AAA-
-TGA-	-TCT-	-TTT-
Pro	Thr	Thr
-CCT-	-ACA-	-ACA-
-GGA-	-TGT-	-TGT-
Ala	Lys	Arg
-GCT-	-AAA-	-AGA-
-CGA-	-TTT-	-TCT-
Ser	Ile	Ile
-TCT-	-ATA-	-ATA-
-AGA-	-TAT-	-TAT-

For all 64 variations:
VIPa analogs when W = Gly
VIPb analogs when W = Gly-Lys-Arg

SUBSTITUTE SHEET

Scheme 2Sequence of the polycloning region of the pPEX vector

	280		284
β-galacto-	IleAspSerIrpIleleu...		
sidase	—AICGATTCITGGATCCCTCTGATGGTACCTCCCTGAGCTCTCTGATGGGCCCGAGTATGGCAGCAGCTGGAATTC—		
	Clal	BamHI	KpnI
			SacI
			ApaI
			EcoRI

SUBSTITUTE SHEET

CLAIMS

1. A method of simultaneously producing a group of individual C-terminally extended analogs of Leu¹⁷-VIP (vasoactive intestinal polypeptide), said group consisting of equally long peptides having the amino acid sequence

5

His-Ser-Asp-Ala-Val-Phe-X-Asp-Asn-Tyr-Thr-Arg-Leu-Y-
 1 2 3 4 5 6 7 8 9 10 11 12 13 14

-Lys-Gln-Leu-Ala-Val-Z-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-
 10 15 16 17 18 19 20 21 22 23 24 25 26 27

-Asn-W
 28 29

15 wherein X represents Thr, Ser, Pro or Ala,
 Y represents Thr, Lys, Ile or Ala
 Z represents Thr, Lys, Ile or Arg, and
 W represents Gly or Gly-Lys-Arg,

20 and of equally long peptides which are also Leu¹⁷-VIP-
 -W²⁹ analogs and which arise from mutations when carrying
 out the method,

c h a r a c t e r i s e d by the steps of
 simultaneously synthesizing a first mixture of
 25 equally long oligonucleotides which during synthesis
 are made ambiguous at the triplets coding for the
 amino acid residues at positions 7 and 14 of Leu¹⁷-VIP,
 and which have a few identical bases at the 3'-end,

simultaneously synthesizing a second mixture
 30 of equally long oligonucleotides which during synthesis
 are made ambiguous at the triplet coding for the amino
 acid residue at position 20 of Leu¹⁷-VIP, and which
 have a few identical bases at the 3'-end, these being
 complementary to the few bases at the 3'-end of the

29

oligonucleotides in the first mixture,

mixing said first mixture with said second mixture, whereby the few bases at the 3'-end of the oligonucleotides of the first mixture anneal to the few bases

5 at the 3'-end of the oligonucleotides of the second mixture, resulting in a mixture of partially double stranded equally long DNA sequences, which thereafter are enzymatically converted into fully double stranded DNA sequences,

10 subjecting said mixture of double stranded DNA sequences, which include at the 3'-end a site for cleavage with a first enzyme and at the 5'-end a site for cleavage with a second enzyme, to cleavage, simultaneously or consecutively, with the first and the
15 second enzyme to produce DNA sequences with 3'- and 5'-ends which are ligatable to the 5'- and 3'-ends of similarly cleaved vectors,

inserting the thus cleaved DNA sequences into the thus cleaved vectors by ligation to produce a
20 mixture of vectors, which are then transformed into hosts in per se known manner,

propagating said hosts to form colonies, which are analyzed one by one to establish those DNA sequences which code for a single protein,

25 propagating separately under expression conditions those hosts which contain vectors having identified protein coding sequences, the C-terminal portions of which correspond to an individual Leu¹⁷-VIP-W²⁹ analog, whereupon the separately expressed proteins
30 are cleaved to release the C-terminal Leu¹⁷-VIP-W²⁹ analogs forming a group of said individual equally long peptides.

2. A method according to claim 1, wherein W represents Gly and the Leu¹⁷-VIP-W²⁹ analogs which arise
35 from mutations when carrying out the method are

30

Asp¹-Ala⁷-Ile¹⁴-Thr²⁰-VIP-Gly²⁹
 Tyr¹-Pro⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Tyr²-Thr⁷-Tyr⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 5 Gly³-Thr⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁴-Thr⁷-Arg¹⁴-Thr²⁰-VIP-Gly²⁹
 Phe⁵-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Asn⁸-Arg¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁷-Gly⁸-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 10 Pro⁷-Gln⁸-Ile¹⁴-Phe¹⁹-Arg²⁰-VIP-Gly²⁹
 Ser⁷-Pro⁸-Ile¹⁴-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Pro¹¹-Lys¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Pro¹²-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-Asn¹⁵-Ile²⁰-VIP-Gly²⁹
 15 Pro⁷-Ile¹⁴-Arg¹⁵-Lys²⁰-VIP-Gly²⁹
 Ala⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Ile¹⁴-Thr¹⁵-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-His¹⁶-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Ile¹⁴-His¹⁶-Thr²⁰-VIP-Gly²⁹
 20 Thr⁷-Ile¹⁴-Phe¹⁹-Ile²⁰-VIP-Gly²⁹
 Pro⁷-Thr¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹
 Ser⁷-Lys¹⁴-Thr²⁰-Ile²⁴-VIP-Gly²⁹
 Thr⁷-Thr¹⁴-Lys²⁰-Asp²⁸-VIP-Gly²⁹
 Gly⁷-Lys¹⁴-Lys²⁰-VIP-Gly²⁹, and
 25 Ala⁷-Asn¹⁴-Arg²⁰-VIP-Gly²⁹.

3. A method according to claim 1, wherein W represents Gly-Lys-Arg and the Leu¹⁷-VIP-W²⁹ analogs which arise from mutations when carrying out the method are

30

Phe²-Pro⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Val³-Thr⁷-Lys¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁴-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Phe⁵-Pro⁷-Thr¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹

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Thr⁶-Ala⁷-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Val⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Ile¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 5 Thr⁷-Arg¹³-Lys¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Gln¹³-Lys¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Arg¹³-Thr¹⁴-Gln¹⁵-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 10 Pro⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Lys¹⁴-Asn¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Asp¹⁹-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Gly¹⁹-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Thr¹⁴-Ile²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 15 Ala⁷-Ile¹⁴-Arg²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Ile¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Ile¹⁴-Thr²⁰-Tyr²⁵-VIP-Gly²⁹-Lys³⁰-Arg³¹ and
 Ser⁷-Thr¹⁴-Ile²⁰-Val²⁶-VIP-Gly²⁹-Lys³⁰-Arg³¹.

20 4. A method according to any one of claims 1-3,
 characterised in that the vectors are
 plasmids and the hosts are E. coli.

5. A C-terminally extended analog of Leu¹⁷-VIP
 characterised in that it is chosen from
 25 the group consisting of peptides having the amino
 acid sequence

His	Ser	Asp	Ala	Val	Phe	X	Asp	Asn	Tyr	Thr	Arg	Leu	Y
1	2	3	4	5	6	7	8	9	10	11	12	13	14

30 -Lys-Gln-Leu-Ala-Val-Z-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-

15	16	17	18	19	20	21	22	23	24	25	26	27
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-Asn-W

35 28 29

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wherein X represents Thr, Ser, Pro or Ala,

Y represents Thr, Lys, Ile or Ala

Z represents Thr, Lys, Ile or Arg, and

W represents Gly or Gly-Lys-Arg,

5 and

- Asp¹-Ala⁷-Ile¹⁴-Thr²⁰-VIP-Gly²⁹
 Tyr¹-Pro⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Tyr²-Thr⁷-Tyr⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 10 Gly³-Thr⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁴-Thr⁷-Arg¹⁴-Thr²⁰-VIP-Gly²⁹
 Phe⁵-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Asn⁸-Arg¹⁴-Arg²⁰-VIP-Gly²⁹
 Pro⁷-Gly⁸-Thr¹⁴-Lys²⁰-VIP-Gly²⁹
 15 Pro⁷-Gln⁸-Ile¹⁴-Phe¹⁹-Arg²⁰-VIP-Gly²⁹
 Ser⁷-Pro⁸-Ile¹⁴-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Pro¹¹-Lys¹⁴-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Pro¹²-Thr¹⁴-Thr²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-Asn¹⁵-Ile²⁰-VIP-Gly²⁹
 20 Pro⁷-Ile¹⁴-Arg¹⁵-Lys²⁰-VIP-Gly²⁹
 Ala⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹
 Ala⁷-Ile¹⁴-Thr¹⁵-Lys²⁰-VIP-Gly²⁹
 Thr⁷-Lys¹⁴-His¹⁶-Lys²⁰-VIP-Gly²⁹
 Pro⁷-Ile¹⁴-His¹⁶-Thr²⁰-VIP-Gly²⁹
 25 Thr⁷-Ile¹⁴-Phe¹⁹-Ile²⁰-VIP-Gly²⁹
 Pro⁷-Thr¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹
 Ser⁷-Lys¹⁴-Thr²⁰-Ile²⁴-VIP-Gly²⁹
 Thr⁷-Thr¹⁴-Lys²⁰-Asp²⁸-VIP-Gly²⁹
 Gly⁷-Lys¹⁴-Lys²⁰-VIP-Gly²⁹
 30 Ala⁷-Asn¹⁴-Arg²⁰-VIP-Gly²⁹
 and
 Phe²-Pro⁷-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Val³-Thr⁷-Lys¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁴-Thr⁷-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 35 Phe⁵-Pro⁷-Thr¹⁴-Ile²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁶-Ala⁷-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹

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Thr⁷-Val⁸-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Thr¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹¹-Ile¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Arg¹³-Lys¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 5 Pro⁷-Gln¹³-Lys¹⁴-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Pro¹³-Thr¹⁴-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ser⁷-Arg¹³-Thr¹⁴-Gln¹⁵-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Lys¹⁴-Gln¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 10 Ser⁷-Lys¹⁴-Asn¹⁵-Arg²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Asp¹⁹-Lys²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Lys¹⁴-Gly¹⁹-Thr²⁰-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Thr⁷-Thr¹⁴-Ile²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Ala⁷-Ile¹⁴-Arg²⁰-Asp²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 15 Ser⁷-Ile¹⁴-Thr²⁰-Lys²⁴-VIP-Gly²⁹-Lys³⁰-Arg³¹
 Pro⁷-Ile¹⁴-Thr²⁰-Tyr²⁵-VIP-Gly²⁹-Lys³⁰-Arg³¹ and
 Ser⁷-Thr¹⁴-Ile²⁰-Val²⁶-VIP-Gly²⁹-Lys³⁰-Arg³¹

wherein all the amino acid residues are of L-configuration.

6. A plasmid containing a gene coding for a C-terminally extended analog of Leu¹⁷-VIP, characterized by a DNA sequence which codes for a C-terminally extended analog of Leu¹⁷-VIP as defined in claim 5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE88/00696

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁸ According to International Patent Classification (IPC) or to both National Classification and IPC ₄ C 12 N 15/00, C 12 P 21/02, C 07 K 7/10, C 07 H 21/00																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="width: 70%; text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top;">IPC 4</td> <td style="vertical-align: top;">C 07 H 21/00 - /04; C 07 K 1/00 - /02; C 07 K 7/10; C 12 N 15/00; C 12 P 21/00 - /02</td> </tr> <tr> <td style="vertical-align: top;">US C1</td> <td style="vertical-align: top;">435:68-71, 170-172.3</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <p style="margin-top: 10px;">SE, NO, DK, FI classes as above. Data base search: WPI/L, CA</p>			Classification System	Classification Symbols	IPC 4	C 07 H 21/00 - /04; C 07 K 1/00 - /02; C 07 K 7/10; C 12 N 15/00; C 12 P 21/00 - /02	US C1	435:68-71, 170-172.3									
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left; padding: 5px;">Category ⁹</th> <th style="width: 70%; text-align: left; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; text-align: left; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top; padding: 5px;">X Y</td> <td style="vertical-align: top; padding: 5px;">EP, A2, 0 184 309 (BEECHAM GROUP PLC) 11 June 1986 See page 3 line 23, page 6 lines 35-38 & JP, 61129198 US, 4737487</td> <td style="vertical-align: top; padding: 5px;">5-6 1-4</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X</td> <td style="vertical-align: top; padding: 5px;">EP, A2, 0 225 020 (BEECHAM GROUP PLC) 10 June 1987 See page 3 line 24 page 5 lines 26-28 & JP, 62116595</td> <td style="vertical-align: top; padding: 5px;">5-6</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X</td> <td style="vertical-align: top; padding: 5px;">US, A, 4 605 641 (DAVID R. BOLIN) 12 August 1986 See column 3, line 63, claim 1 & US, 4734400</td> <td style="vertical-align: top; padding: 5px;">5-6</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="vertical-align: top; padding: 5px;">WO, A1, 85/02198 (AMGEN) 23 May 1985 & EP, 0150572 JP, T, 61500250 .../...</td> <td style="vertical-align: top; padding: 5px;">1-4</td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X Y	EP, A2, 0 184 309 (BEECHAM GROUP PLC) 11 June 1986 See page 3 line 23, page 6 lines 35-38 & JP, 61129198 US, 4737487	5-6 1-4	X	EP, A2, 0 225 020 (BEECHAM GROUP PLC) 10 June 1987 See page 3 line 24 page 5 lines 26-28 & JP, 62116595	5-6	X	US, A, 4 605 641 (DAVID R. BOLIN) 12 August 1986 See column 3, line 63, claim 1 & US, 4734400	5-6	Y	WO, A1, 85/02198 (AMGEN) 23 May 1985 & EP, 0150572 JP, T, 61500250 .../...	1-4
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">1989-02-21</td> <td style="border-bottom: 1px solid black; padding: 5px;">1989-03-13</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">Swedish Patent Office</td> <td style="padding: 5px;"> Yvonne Siösteen </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	1989-02-21	1989-03-13	International Searching Authority	Signature of Authorized Officer	Swedish Patent Office	 Yvonne Siösteen							
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A1, 0 194 006 (IMPERIAL CHEMICAL INDUSTRIES PLC) 10 September 1986 & JP, 61275300	1-4

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